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Importance of a Small N-Terminal Region in Mammalian Peptide Transporters for Substrate Affinity and Function

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Abstract. The two closely related, proton-coupled, electrogenic mammalian peptide transporters PEPT1 and PEPT2 differ substantially in substrate affinity and mode of function. The intestinal carrier PEPT1 has a lower affinity for most substrates than the isoform PEPT2 that is expressed in kidney, lung, brain and other tissues. A previous analysis of PEPT1-PEPT2 chimeras has suggested that the N-terminal half of the carrier proteins is important for substrate affinity. We constructed and analyzed new PEPT1-PEPT2 chimeras for identifying smaller segments within the N-terminal region of the transporter proteins that contribute to the kinetic properties. The first 59 or 91 amino-acid residues of PEPT1 were used to replace the corresponding region in PEPT2 leading to the chimeras CH3 and CH4, which could be analyzed when expressed in *Xenopus laevis* oocytes. Substrate affinities of both chimeras for the zwitterionic substrate D-Phe-Ala ranged between those that are characteristic for either PEPT1 or PEPT2, but when charged dipeptide substrates were employed, both chimeras possessed PEPT1-like affinities. The chimera CH3 carrying the N-terminal 59 amino-acid residues of PEPT1 exhibited a PEPT2-like phenotype with respect to pH_{out}-dependency as well as to the current-voltage relationship of inward currents. In the chimera CH4 possessing the 91 amino-terminal residues of PEPT1, a pronounced alteration in the pH_{out}-dependence was observed, with highest transport rates occurring at pH values as low as pH 4.0. Based on this analysis, we propose that the two identified aminoterminal regions in mammalian peptide carriers play an important role in determining the substrate affinity and also other characteristic features of the two transporter subtypes.

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Introduction

The cellular uptake of dipeptides and tripeptides across the apical membrane of intestinal and renal tubular cells is mediated by specific electrogenic, proton-coupled cotransporters. The cDNAs encoding the intestinal and kidney peptide transporter PEPT1 and PEPT2 have been cloned from different species [5, 6, 13, 18, 19, 23, 24] and more recently, the structures of mouse PEPT1 and PEPT2 genes have been described [15, 22]. The open reading frames of PEPT1 and PEPT2 predict gene products of 707–710 and 729 amino-acid residues, respectively, with several putative glycosylation and phosphorylation sites. Hydropathy analysis of primary sequences and an epitop-insertion approach [9] suggested that the proteins contain 12 transmembrane domains (TMD), with both the C- and N-termini localized inside the cell. The overall sequence identity between PEPT1- and PEPT2-subgroups along the different species is approximately 50%. The amino-acid sequence in the intra- or extracellular loops is more divergent than that in the putative TMDs. In vitro translation of the cRNAs in the presence of reticulocyte lysate revealed core glycosylated gene products of 71 kDa for rabbit PEPT1 and 107 kDa for rabbit PEPT2, indicating also different degrees of glycosylation [6, 13]. While PEPT1 is predominantly expressed in the intestine with lower expression levels in kidney, PEPT2 is found in a variety of tissues including brain and mammary gland [3, 6, 24] and more recently evidence for the presence of PEPT2 in bronchial epithelial cells, type II pneumocytes and in cells of the choroid

plexus has been obtained by immunohistochemistry and by functional studies [16, 30].

The substrate specificities and kinetic properties of PEPT1 and PEPT2 have been extensively studied in heterologous expression systems with *Xenopus laevis* oocytes, mammalian cells and yeast cells by flux studies and/or electrophysiological techniques [1, 2, 11, 12, 20, 21, 26]. PEPT1 as well as PEPT2 are able to transport a huge variety of different zwitterionic, anionic and cationic dipeptides, tripeptides as well as peptidomimetics in an electrogenic mode as a consequence of H_3O^+/substrate cotransport. Whereas PEPT1 couples transport of one substrate molecule to one H_3O^+, PEPT2 requires two protons for coupling [7, 26]. Besides these differences in substrate-ion flux coupling stoichiometry, other transport properties of PEPT1 and PEPT2 are also quite different. PEPT1 shows a lower affinitiy than PEPT2 for almost all substrates tested. In addition, the transmembrane pH gradient and the membrane potential affect the transport activities of PEPT1 and PEPT2 differently, allowing these parameters to be used for identification of functionally important protein segments.

Despite several studies on the transport mechanism of the two peptide transporters, little is known about the structural elements that are responsible for the functional properties of PEPT1 and PEPT2. It has been shown that conserved histidine and tyrosine residues in the second, fourth and fifth transmembrane domain (TMD) of the carrier proteins are essential for activity with involvement in either proton and/or substrate binding [4, 8, 14, 28, 31]. Moreover, studies with PEPT1/PEPT2 chimeras suggested that the first half of the carrier proteins (up to residue 401) may contain segments that form the substrate binding domain and determine other functional properties as well [10, 14, 29]. In the present study, we constructed and phenotyped new PEPT1-PEPT2 chimeras with different N-terminal segments of PEPT1 replacing the same regions in a PEPT2 backbone in order to map smaller protein segments that could be involved in substrate binding and kinetics characteristics within the peptide transporters.

Methods and Materials

Plasmid Constructions and Amino-Acid Numbering of Chimeras

The *E. coli* strain XL-1 Blue (Stratagene, Heidelberg, Germany) was used for transformation and propagation of the recombinant plasmids. Cloning procedures were essentially performed as described by Sambrook et al. [25]. For all mutated or chimeric constructs the rabbit PEPT1 (2.7 kb) or rabbit PEPT2 (4.2 kb) cDNA was inserted between the *Sal*I and *Not*I sites of the plasmid pSPORT1 [5, 6]. Chimeras generated by PCR were validated by sequence analysis prior to functional studies. Amino acid numbering refers to the intestinal isoform PEPT1.

CH2 Construct

The construction of the plasmid used, pSPORT1-PEPT2^{ClaI}; containing a *Cla*I site in codon 402 of the PEPT2 cDNA, was been described elsewhere [10]. To generate CH2, a 1.2-kb *Sal*I-*Cla*I fragment from the PEPT1 cDNA encoding the first 401 amino acids was inserted between the corresponding *Sal*I and *Cla*I sites of the plasmid pSPORT1-PEPT2^{ClaI}. The resulting construct CH2 encodes a transporter consisting of amino-acid residues 1–401 of PEPT1 and 402–707 of PEPT2.

CH3 Construct

By using the site-directed mutagenesis system from Pharmacia ("two-primer system"; U.S.E. Mutagenesis Kit, Heidelberg, Germany) with the primers f1 (mutagenesis primer) and b1 (selection primer) a *Xho*I-site in the codons 90/91 of the PEPT2-cDNA was created. The resulting plasmid was named pSPORT1-PEPT2^{XhoI}. The chimera was then constructed by replacing the first 276 nucleotides of the open reading frame of PEPT2^{XhoI} with a fragment encoding the corresponding sequence of PEPT1. Therefore the PCR primers f2 and b2, which incorporated *Sal*I and *Xho*I sites into the ends of the fragment, were used to amplify the appropriate sequence from the PEPT1-cDNA. The PCR product was digested by the two enzymes and ligated into the *Sal*I and *Xho*I sites of pSPORT1-PEPT2^{XhoI}. The resulting construct CH3 encodes a transporter with amino acid residues 1–59 of PEPT1 and 60–707 of PEPT2.

CH4 Construct

For constructing CH4, the elimination of the *Ava*I site in the polylinker region between the *Kpn*I and *Sal*I site of pSPORT1-PEPT1 was necessary. For this purpose a synthetic linker (elimination of *Ava*I), which was derived from the primers f3 and b3, was inserted into the *Kpn*I-*Sal*I sites of pSPORT1-PEPT1. The resulting plasmid was named pSPORTI-(*Ava*I-PEPT1. Next, a synthetic linker derived from primers f4 and b4 was ligated between the *Acc*I-*Pml*I sites of the plasmid pSPORT1-(*Acc*I)-PEPT1. The resulting plasmid was named pSPORT1-CH7. Finally, chimera CH4 was generated by inserting an *Ava*I fragment from pSPORT1-CH7 into the *Ava*I sites of pSPORT1-PEPT2. The resulting construct CH4 contains the coding sequence of PEPT1 (residues 1–91) and PEPT2 (92–707).

Xenopus laevis Oocytes and Transport Assay

Oocyte preparation, maintenance and injection techniques have been described previously [5]. Capped complementary RNA (cRNA) was transcribed in vitro from *Not*I-linearized cDNA of peptide transporters by using the T7 RNA polymerase kit from Ambion (Austin, TX). Three days (18°C) after injection of 13.6 ng cRNA of the transporters, uptake of ^{3}H-D-Phe-Ala or ^{3}H-D-Phe-Glu was performed at standard assay conditions in a buffer composed of (in mM) 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 5 MES/Tris (pH 3.5 to 6.5) or HEPES/Tris (pH 7.0 to 8.5). Kinetics of ^{3}H-D-Phe-Ala influx at pH 6.0 were measured for 30 min of incubation in the presence of increasing concentrations of substrate (0.05, 0.1, 0.2, 0.4, 1.0, 2.5, 5.0, 10 mM for PEPT1; CH3 and CH4; 0.005, 0.01, 0.02, 0.04, 0.1, 0.25, 0.5, 1.0 for PEPT2). Corresponding uptake rates in water-injected control oocytes were subtracted. Inhibition of ^{3}H-D-Phe-Ala (5 μM) at pH 6.0 in the presence of increasing concentrations of competitors (0.1, 0.2, 0.4, 1.0, 2.5, 5.0, 10 mM for PEPT1, CH3 and CH4; 0.005, 0.01, 0.02, 0.04, 0.1, 0.25, 0.5, 1.0 for PEPT2) was determined for Gly-Glu (glycyl-L-glutamate) and Gly-Lys (glycyl-L-lysine).

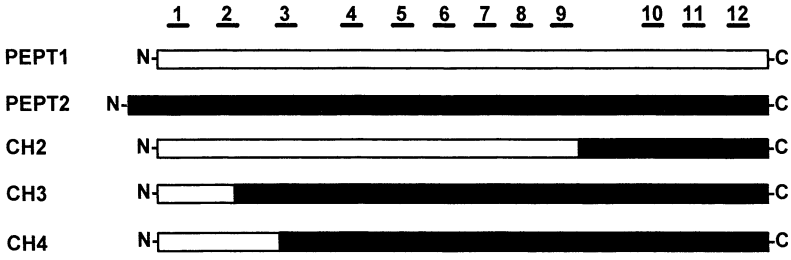

Fig. 1. Schematic representation of wild-type and chimeric peptide transporters. Shown are schematic representations of wild-type and chimeric peptide transporters with 12 putative transmembrane domains (1–12) whereby the amino (*N*) and carboxy (*C*) termini are cytosolic. Regions of proteins comprised of the PEPT1 sequence are shown in white, and regions comprised of PEPT2 are shown in black.

Electrophysiology

A conventional two-electrode voltage-clamp technique was applied to characterize responses in current (*I*) and transmembrane potential (V_m) to substrate addition in oocytes injected with 13.6 ng cRNA of the transporter isoforms as described previously [5]. Steady-state current-voltage (*I-V*) relationships were measured in the absence and the presence of substrates, with water-injected oocytes serving as controls. Membrane potential in oocytes was held at −60 mV and stepped symmetrically to test potentials from −120 mV to +40 mV by 500 msec rectangular voltage pulses, current recordings were obtained during the last 100 msec.

Calculations

All calculations were performed by using INPLOT, statistical analysis by INSTAT (GraphPAD, Los Angeles, CA). Flux studies as well as most of the electrophysiological experiments were carried out with 6–10 oocytes from at least two separate batches and results are presented as the mean ± SEM.

Materials

Peptides were purchased from Sigma (Deisenhofen, Germany]. Custom-synthesized ^{3}H-D-phenylalanyl-L-alanine (^{3}H-D-Phe-Ala; 22.5 Ci/mmol) and ^{3}H-D-phenylalanyl-L-glutamate (^{3}H-D-Phe-Glu; 12 Ci/mmol] were obtained from Zeneca (Billingham, UK). The following oligonucleotides (Eurogentec, Seraing, Belgium) were used:

f1: 5′-CTGTCTACCATGCCTTCTCGAGCCTCTGCTATTTCACTCCC-3′; b1: (5′-CCGCTCTAGAGGATCCAAGCTAGCGTACGTGCATGCG-3′; f2: 5′-CCGGTCCGGAATTCCCGGGTCGACCCACGCGTCG-3′; b2: 5′-AATGGGCGTGAGGTAGCACAGGCTCGAGAACGTGTGGTAGATGACCG-3′; f3: 5′-TCGACCGGGGAATTCCGGACCCGTAC-3′; b3: GGGTCCGGAATTCCCCGG-3′; f4: 5′-CTACGTGCTTGGCCATGTGATCAAGTCCT TAAGCGCATTTCCAATACTCGGGGGGAAAGTGGTACAC-3′; b4: 5′-GTGTACCACTTTCCCCCCGAGTATTGGAAATGCGCTTAAGGACTTGATCACATGGCCAAGCACGT-3′.

Results

Expression of Chimera CH2 in *Xenopus laevis* Oocytes

The nomenclature and composition of constructed chimeras is shown schematically in Fig. 1. Chimeras (CH2 to CH4) were composed of PEPT1 from the N-terminus to the ninth (CH2), third (CH4) or second (CH3) TMD and PEPT2, from these points to the C-terminus. Transport activity of wild-type and chimeric peptide transporters was determined after expression in *Xenopus laevis* oocytes by flux studies (5 μM ^{3}H-D-Phe-Ala) and electrophysiology (5 mM Gly-Gln). As shown in Table 1, only the chimera CH2 failed to mediate influx of ^{3}H-D-Phe-Ala and lacked of transport currents when analyzed in the voltage-clamp mode. However, a GFP-tagged fusion protein of CH2 allowed the protein expression to be determined by confocal scanning laser microscopy and localized the protein to the cell membrane of oocytes (*not shown*). The principal defect of the CH2 protein therefore appears to be a complete lack of transport function rather than a trafficking and/or insertion defect.

Substrate Affinities of the Chimeras CH3 and CH4

For a functional characterization of dipeptide transport by the chimeras CH3 and CH4 we performed a kinetic analysis by flux studies and determined the apparent K_m for uptake of our model dipeptide D-Phe-Ala. As shown in Table 2, the affinities of chimeras CH3 and CH4 for the zwitterionic substrate D-Phe-Ala are between wild-type PEPT1 and PEPT2. Since the phenotype of the chimeras might be dependent on the particular substrate used, we determined also the affinities of CH3 and CH4 to other substrates. This was done by inhibiton of ^{3}H-D-Phe-Ala influx into PEPT1-, PEPT2-, CH3- and CH4-expressing oocytes in the presence of increasing concentrations of differently charged glycyl-peptides. Interestingly, the EC_{50} values (Table 2) obtained from the competition curves show that the affinities of PEPT1, CH3 and CH4 for anionic dipeptides are very similar and approximately twentyfold lower than those of PEPT2. Affinities of CH3 and CH4 for a positively charged dipeptide are about 1.3 times higher than that of PEPT1. However, in comparison to PEPT2, the chimeras CH3 and CH4 as well as PEPT1 possess a fivefold lower affinity to cationic dipeptides.

Since the affinities of charged substrates—in particular of anionic peptides—for PEPT1 and

Table 1. Functional expression of wild-type and chimeric peptide transporters.

Transporters	Influx (D-Phe-Ala) [pmol/30min/oocyte]	Inward current (Gly-Gln) [nA]
PEPT1	2.71 ± 0.24	230.0 ± 4.5
PEPT2	6.34 ± 0.45	40.0 ± 2.1
CH2	0.12 ± 0.02	1.1 ± 0.8
CH3	3.79 ± 0.45	25.1 ± 4.5
CH4	2.22 ± 0.13	14.1 ± 0.5

Oocytes injected with the cRNA of wild-type (PEPT1, PEPT2) or chimeric transporters (CH2 to CH4) were used for measurements. Corresponding transport rates in water-injected oocytes were subtracted. Influx of 5 μM ^{3}H-D-Phe-Ala into oocytes was measured at pHout 6.0 after 30 min of incubation. Steady-state inward currents were measured by the two-electrode voltage-clamp technique. Oocytes were perfused with 5 mM Gly-Gln at pH 6.0 and substrate-evoked inward currents were recorded while the membrane potential was clamped to −60 mV. Data represent the mean ±SEM for 8–10 oocytes per condition and are representative for at least two similar experiments.

Table 2. Apparent affinities of wild-type and chimeric peptide transporters.

Transporters	D-Phe-Ala App.K_m[μM]	Gly-Lys EC_{50}-value [μM]	Gly-Glu EC_{50}-value [μM]
PEPT1	1368 ± 361	840.9 ± 49.4	226.9 ± 64.0
PEPT2	58.5 ± 11.1	135.1 ± 8.6	11.3 ± 1.7
CH3	239.8 ± 12.3	697.3 ± 57.5	190.2 ± 38.2
CH4	283.4 ± 34.1	647.5 ± 163	253.3 ± 19.7

Uptake of ^{3}H-D-Phe-Ala into oocytes expressing PEPT1, PEPT2, CH3 or CH4 was measured over 30 min of incubation at pH_{out} 6.0 in the presence of increasing concentrations (*see* Methods) of D-Phe-Ala, Gly-Lys or Gly-Glu. Uptake rates in oocytes injected with water were subtracted. The obtained uptake rates were transformed according to Eadie-Hofstee and apparent K_m values were derived by linear regression analysis by the least-square method (r^2: in all cases ≥ 0.95). EC_{50}-values were derived by the least-square method based on a competition curve with one component (r^2: in all cases ≥ 0.97). Data represent the mean ±SEM for repeated measurements in two batches oocytes.

PEPT2 were found to depend strongly on membrane potential [1, 2], we extended the comparative analysis of substrate affinities of wild-type and chimeric transporters by using the two-electrode voltage-clamp method and employed Gly-Glu as a representative negatively charged substrate. Inward currents measured as a function of substrate concentration were used to determine the substrate affinities at different membrane potentials. As shown in Table 3, the affinity of CH3 for Gly-Glu changed, similarly to that of PEPT1, with alterations of membrane potential. Apparent K_m values were approximately 15–20 times lower than those of PEPT2 and decreased at hyperpolarized membrane potentials. This low, PEPT1-like affinity confirmed the obtained low affinity based on the EC_{50} value for Gly-Glu in the tracer-flux study. In the case of CH4, the substrate-evoked inward currents measured at membrane potentials between −20 to −60 mV were extremely low (see Fig. 2) and kinetic analysis could only be performed at hyperpolarized membrane potentials. As demonstrated in Table 3, apparent K_m values obtained for CH4 were similar to those of CH3 and PEPT1. From this part of the studies it is apparent that the exchanged aminoterminal regions up to the second (CH3) or third (CH4) transmembrane domain possess regions that are of key importance for determining the substrate affinity of PEPT1.

I-V Relationship of the Chimeras CH3 and CH4

Because the membrane potential affects the transport activities of wild-type transporters differently, *I-V* relationships of CH3 and CH4 were recorded to investigate whether the membrane-potential dependency of the chimeras is also altered. As shown in Fig. 2, in a representative *I-V* relationship, maximal currents evoked by Gly-Glu in the case of PEPT1 exceeded those of PEPT2, CH3 and CH4. Moreover, whereas PEPT1 showed its typical *I-V* relationship with positive inward currents already at low membrane voltages, CH3 and CH4 possessed a PEPT2 phenotype with increasing inward currents only at high hyperpolarizing membrane potentials (> −80 mV).

Transport Acitivity of CH3 and CH4 as a Function of Extracellular pH

Besides substrate affinity and *I-V* relationship, PEPT1 and PEPT2 also differ with respect to the pH-dependent transport activity [5, 6] at low substrate concentrations. We therefore extended the comparative analysis of chimeras CH3 and CH4 by determining transport activity as a function of pH_{out} for both, the zwitterionic (D-Phe-Ala) and the anionic dipeptide D-Phe-Glu (Fig. 3). Highest uptake rates

Table 3. Apparent affinities of wild-type and chimeric peptide transporters at different membrane potentials assessed by Gly-Glu.

Membrane potential [mV]	Apparent K_m assessed by Gly-Glu [μM]			
	PEPT1	PEPT2	CH3	CH4
0	50.4 ± 5.7	9.04 ± 1.3	125 ± 20.4	n.d.
−20	75.9 ± 10.3	10.4 ± 1.8	80.4 ± 8.9	n.d.
−40	170 ± 17.3	11.4 ± 1.9	83 ± 5.9	n.d.
−60	256 ± 20.3	12.8 ± 2.4	124 ± 30.4	n.d.
−80	322 ± 28.4	14.5 ± 2.2	173 ± 30.0	967 ± 340
−100	409 ± 30.8	25.9 ± 5.4	303 ± 74.1	748 ± 371
−120	650 ± 50.4	40.4 ± 10.3	428 ± 80.4	766 ± 354

Oocytes expressing PEPT1, PEPT2, CH_3 or CH_4 were perfused at pH_{out} 6.0 in the presence of increasing Gly-Glu concentrations (*see* Methods). At each substrate concentration, steady state *I-V* relationships were recorded by the two-electrode voltagte-clamp technique and currents obtained were replotted as a function of substrate concentration. The obtained apparent K_m values were then plotted against the membrane potential. n.d. not determined. Data represent the mean ± SEM and are representative at least two similar experiments.

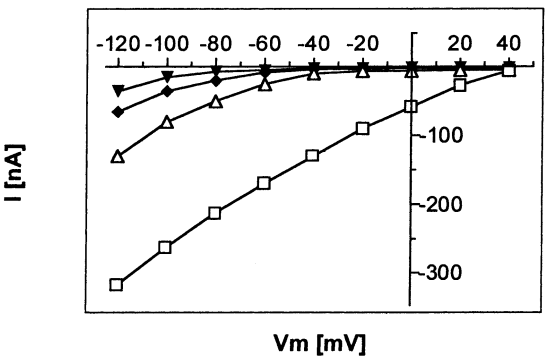

Fig. 2. Transport activity as a function of membrane potential of wild-type and chimeric peptide transporters. Recordings of *I-V* relationship in oocytes expressing PEPT1 (□), PEPT2 (Δ), CH3 (◆) or CH4 (▼) superfused with 5 mM Gly-Glu. V_m was stepped symmetrically to the test potentials between +40 mV and −120 mV in steps of 20 mV and current response in the presence of 5.0 mM Gly-Glu, subtracted by the current in the absence of substrate, were plotted as function of V_m at pH_{out} 6.0. Data represented are typical recordings as obtained in at least two similar experiments.

were obtained for both dipeptides at pH_{out} 6.5 (PEPT1) or pH_{out} 5.5 (PEPT2) with the characteristic differences in the pH_{out} dependence between the two transporter types. Whereas CH3 showed with both substrates a PEPT2-like phenotype, in the case of CH4, extremely low pH_{out} values (pH 4.0 to 4.5) were needed for optimal transport activity.

Discussion

Employing two chimeras with aminoterminal stretches of PEPT1 replacing the same regions of PEPT2 within a PEPT2 backbone revealed that this segment comprising the first 91 amino acids is involved in substrate-binding characteristics and transport functions. The two chimeric transporters characterized here allow some conclusions to be drawn about the possible roles of the aminoterminal region including the first, second and third TMD in the mammalian peptide transporters. It needs to be emphasized that the conclusions are based on altered functional characteristics of the proteins rather than on a loss of function often associated with mutations introduced to address the importance of individual amino-acid residues [4, 8, 14, 28, 31]. There are also limitations of the chimeric approach, the identified protein regions do not necessarily correspond to the entire functional domain but may only form a part of it. Nevertheless, phenotype analysis of either mutated transporters or chimeras helps to define the structure-function relationship of mammalian peptide transporters.

Previous investigations of PEPT1-PEPT2 chimeras [10, 14, 29] have shown that the N-terminal halves of both transport proteins contain the regions that determine the substrate specificity and the other functional features. When the large, more than 200 amino-acid-containing extracellular loops between TMD 9 and TMD 10 and the remaining C-terminal section of PEPT1 were fused onto the aminoterminal half of PEPT2, the resulting chimera possessed all the PEPT2-specific characteristics. Here we show that the reverse construct (CH2), comprised of PEPT1 up to TMD 9 and PEPT2 towards the carboxy terminal end, failed to induce any measurable transport activity when expressed in oocytes, although a GFP-CH2-fusion protein allowed its presence in the oocyte membrane to be determined. Why this chimera is inactive remains to be determined. However, when only the first 59 (CH3) or 91 (CH4) amino-acid residues derived from PEPT1 were exchanged with the remaining sequence represented by PEPT2, functional analysis of the chimeras was possible.

CH3 displayed substrate affinities for the zwitterionic substrate D-Phe-Ala about five times lower than those of PEPT2 but also 5 times higher than those of PEPT1. In case of the charged substrates (Gly-Lys, Gly-Glu), however, typical PEPT1-like affinities were obtained. From the functional analysis

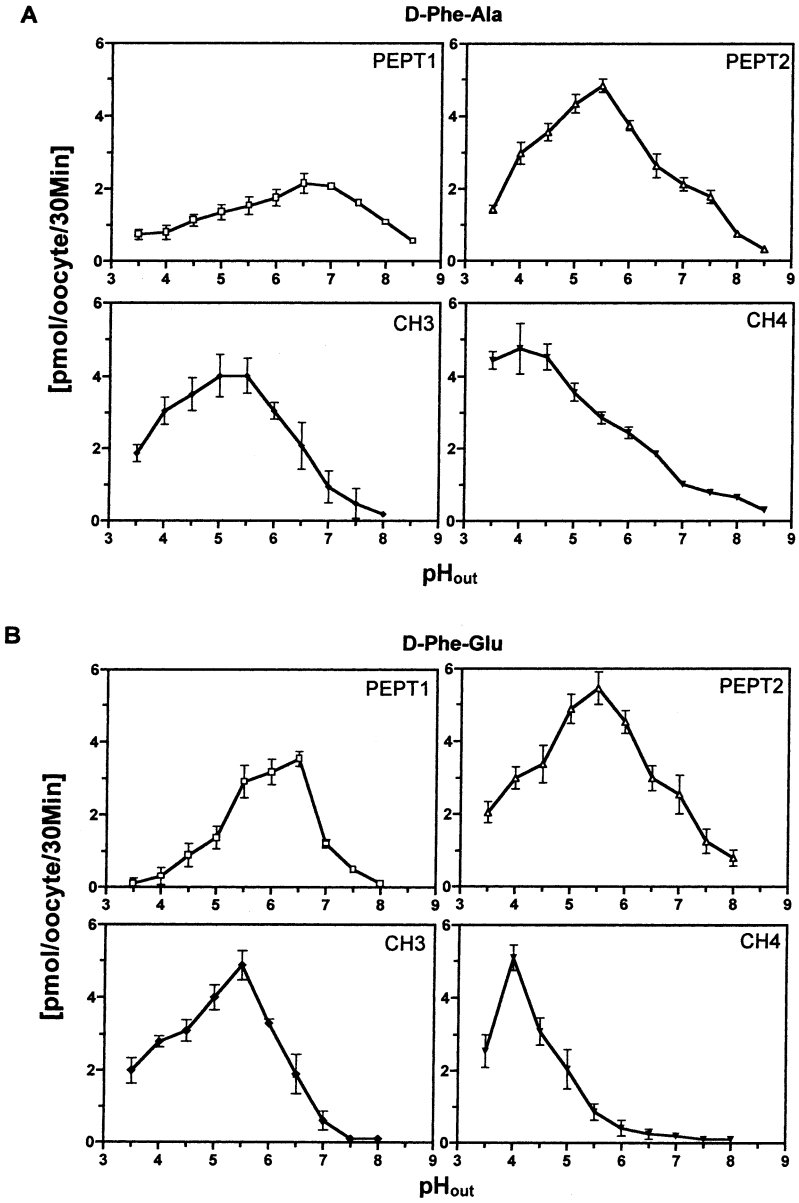

Fig. 3. pH_{out}-dependent transport activity of wild-type and chimeric peptide transporters. Influx of 5 μM ^{3}H-D-Phe-Ala (***A***) or 5 μM ^{3}H-D-Phe-Glu (***B***) into oocytes expressing PEPT1 (□), PEPT2 (Δ), CH3 (◆) or CH4 (▼) was measured for 30 min of incubation at different extracellular pH values (pH_{out}). Corresponding uptake rates of ^{3}H-D-Phe-Ala or ^{3}H-D-Phe-Glu into oocytes injected with water were subtracted. Uptake rates represent the mean ± SEM for 8–10 oocytes per condition and are representative of at least two similar experiments

of CH3, it becomes apparent that the first 59 amino-acid residues of PEPT1 substantially influence its substrate affinity. It needs to be emphasized that our conclusion is only applicable to PEPT1 but not to PEPT2, since a chimera possessing N-terminal residues of PEPT2 on a PEPT1 backbone was not analyzed. Nevertheless, the phenotype of CH3 implicates that the exchanged N-terminal segment of PEPT1 plays a role in the interaction of the side chains within the substrates with the protein and that this interaction largely determines substrate affinity. This proposal of substrate side-chain interactions with the binding domain is supported by crystallographic and calorimetric analysis of ligand binding to an oligopeptide transporter-protein complex from *E. coli* [27]. There, it has been shown that the aminogroup and the carboxygroup, as well as the side chains of a number of peptides, form strong and specific hydrogen bonds with the hydrated binding pocket of the protein complex. However, the different affinity constants of the substrates to the transporter complex were explained by interaction between the ionic or nonionic side chains of substrates to the binding region of the protein. Assuming an evolutionary conservation of ligand binding by peptide transporter proteins, the aminoterminal region up to the second TMD of PEPT1 could be involved in accomodating the side chains of charged peptide substrates and consequently determine binding affinity to this particular substrate class.

When transport activity of CH3 was determined as a function of pH_{out}, CH3 possessed a PEPT2 phenotype with a much more pronounced increase in transport rate by lowering pH_{out} from 8.0 to 5.5 than

in the case of PEPT1. It needs to be stressed here that the differences in pH_{out} effects on PEPT1 and PEPT2 can only be determined at lower substrate concentrations and that the pH profiles also depend on the substrate used. This becomes evident by the more bell-shaped pH profile of PEPT1 when D-Phe-Glu was used as a substrate compared to the flatter pH profile in the case of D-Phe-Ala. Nevertheless, CH3 conserved the PEPT2 phenotype in the pH_{out} dependence also with the anionic substrate, even with an apparent PEPT1-like K_m for the charged substrate. It therefore appears that the aminoterminal segment of PEPT1 is responsible neither for its characteristic *I-V* relationship nor its pH_{out}-dependency.

As far as the substrate binding pocket in the mammalian peptide transporters is concerned, recent reports using chimeras [10, 14, 29] and mutated peptide transporters [22–26] have suggested that amino acids from TMD 1, TMD 4 with H121, TMD 5 with Y167, as well as the region from TMD7 to TMD9, may contribute to the substrate-binding domain. These results together with our present findings are consistent with a molecular modeling approach [4], in which the authors predict the substrate-binding domain of human PEPT1 to be formed by Y12 and E26 from TMD1, Y91 from TMD3, Y167 from TMD5, W294 and R282 from TMD7, D341 from TMD8 and Y588 and E595 from TMD10. From the current analysis we can conclude that although residues Y12 and E26 from TMD1 may be important for overall substrate binding, they most likely do not contribute to the differences in substrate affinity of PEPT1 and PEPT2, as they are embedded in a highly conserved stretch that has been exchanged here and that caused the marked changes in affinity seen in CH3.

Investigations of protein regions or amino-acid residues that may confer the pH_{out} dependency of transport activity of the peptide transporters are limited to two studies [8, 10]. A chimera consisting of PEPT1 with the first 9 TMD derived from PEPT2 had a pH_{out} dependency almost identical to PEPT2, suggesting an involvement of the first 9 TMD in the pH_{out} dependency. Mutational analysis of histidine residues assumed to serve as proton acceptors in PEPT1 showed that the histidine at position 121 replaced by either R or C displayed only very minor changes in the pH_{out} dependence when compared to wild-type PEPT1. However, the histidine residue at position 57 was found to be essential, as transport function was lost when the histidine was replaced by other amino acids. With the chimera CH4 we have generated the first severely altered but fully functional pH_{out}-phenotype, in which transport of D-Phe-Ala and D-Phe-Glu occurred with pH_{out} optima at pH 4.5 and 4.0. So far, the decline in transport activity observed for wild-type transporters at $pH < 6$ for PEPT1 and $pH < 5.5$ for PEPT2 (see also Fig. 3) has been considered to represent a progessive denaturation by acid exposure. In case of CH4, such an inactivation could only be detected at pH_{out} 3.5 in the case of the anionic dipeptide. Even more impressive is that CH4 displayed at pH_{out} 3.5 a maximal transport rate with D-Phe-Ala that was not significantly different from that of wild-type PEPT2 at pH_{out} 5.5. From the pH_{out}-profiles provided in Fig. 3 it can be calculated that the half-maximal stimulation of transport of D-Phe-Ala and D-Phe-Glu by wild-type PEPT2 is reached at an apparent external H^+ activity of 200 to 250 nM. In contrast, CH4 requires a 10-fold (D-Phe-Ala) or even a 100-fold (D-Phe-Glu) higher external H^+ activity to reach half-maximal transport activation.

This extreme pH_{out} dependency of CH4 is not observed with CH3. Therefore, differences in the primary structure of CH3 and CH4 and particularly amino-acid residues 60 to 91 appear to be responsible for their different phenotypes. It needs to be emphasized here that this region cannot explain the difference in pH_{out} dependency between wild-type transporters, but interaction of this domain with other protein regions could be critical for pH_{out}-dependent transport activity. Inspection of the amino-acid residues 60 to 91 of PEPT1 and PEPT2 may reveal some clues regarding which residues may be important for such an interaction for pH_{out} sensing. PEPT1 contains amino acids ^{60}VA^{61} in the second TMD. These residues are substituted in PEPT2 by the amino acids ^{60}SS^{61}. Also within the second TMD, the threonine T66 in PEPT1 is substituted by a phenylalanine in PEPT2. More subtle substitutions are found at position 85 (V to I), 86 (W to Y), 88 (I to L), 90 (Y to N) and 91 (T to V). Any of these amino-acid residues could be involved in the pH_{out} dependence by interacting with residues in another region of the transporter protein.

In summary, we identified a discrete region (amino-acid residues 1–59) that contributes significantly to the different substrate affinities found in the mammalian peptide transporters. The first 59 amino acid residues up to the second. TMD may, therefore, form an important part of the substrate-binding domain in peptide transporters that is also important for the interaction of side chains of substrates within the protein. Furthermore, by analysis of the chimera CH4, we obtained indirect evidence that the region between the centers of the second and third TMD (amino acid residues 60–91) contributes significantly to the characteristic pH_{out}-dependency of transport function in peptide transporters.

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